Modes of Operation and Variable Stoichiometry of the Furosemide-sensitive Na and K Fluxes in Human Red Cells

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ABSTRACT We report in this paper different modes of Na and K transport in human red cells, which can be inhibited by furosemide in the presence of ouabain. Experimental evidence is provided for inward and outward coupled transport of Na and K, K_i/K_o and Na_i/Na_o exchange, and uncoupled Na or K efflux. The outward cotransport of Na and K was defined as the furosemide-sensitive (FS) component of Na and K effluxes into choline medium and as the Cl-dependent or *cis*-stimulated component of the ouabain-resistant (OR) Na and K effluxes. Inward cotransport of Na and K was defined by the stimulation by external Na (Na_o) of the K influx and the stimulation by external K (K_o) of the Na influx in the presence of ouabain. Both effects were FS and Cl dependent. Experimental evidence for an FS K_i/K_o exchange pathway of the Na/K cotransport was provided by (*a*) the stimulation by external K of FS K influx and efflux, and (*b*) the stimulation by internal Na or K of FS K influx in the absence of external Na. Evidence for an FS Na_i/Na_o exchange pathway was provided by the stimulation of FS Na influx by internal Na from a K-free medium (130 mM NaCl). This pathway was four to six times smaller than the K_i/K_o exchange. In cells containing only Na or K, incubated in media containing only Na or K, respectively, there was FS efflux of the cation without simultaneous inward transport (FS uncoupled Na and K efflux). The stoichiometric ratio of FS outward cotransport of Na and K into choline medium varied with the ratio of Na_i-to-K_i concentrations, and when Na_i/K_i was close to 1, the ratio of FS outward Na to K flux was also 1. In choline media, FS Na efflux was inhibited by external K (noncompetitively), whereas FS K efflux was stimulated. The stimulation of FS K efflux was due to the stimulation by K_o of the K_i/K_o exchange pathway. Thus, the stoichiometry of FS Na and K effluxes also varied in the presence of external K. A minimal model for a reaction scheme of FS Na and K transport accounts for *cis* stimulation, *trans* inhibition, and *trans* stimulation, and for variable stoichiometry of the FS cation fluxes.

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INTRODUCTION

In recent years, it has been shown that several epithelial and red cells possess cotransport systems such as Na/K/Cl, Na/Cl, and K/Cl, which appear to be involved in the secretion or absorption of these ions and in volume regulation. An Na/K/Cl cotransport system has been demonstrated in human red cells (Wiley and Cooper, 1974), in duck red cells (Schmidt and McManus, 1977*a*, *b*), and in many other cell types (for a review, see Palfrey and Rao, 1983). An Na/Cl cotransport system has been characterized in brush border of flounder intestine (Frizzel et al., 1979), in the mouse thick ascending limbs of Henle (Hebert et al., 1981), and in the urinary bladder of the winter flounder (Stokes, 1984). A K/Cl cotransport has been shown in low-K sheep red cells (Dunham and Ellory, 1981) and in the basolateral membrane of *Necturus* gallbladder (Reuss, 1983). K/Cl cotransport appears to be activated by treatment with the sulfhydryl group reagent *N*-ethylmaleimide in low-K sheep red cells (Lauf, 1983) and in human red cells (Lauf et al., 1984).

These three types of cotransport systems have a common specific linkage to Cl ions and inhibition, but with different K_i, by the loop diuretics bumetanide and furosemide. Attempts have been made to distinguish between these different types of cotransport by studying their pharmacological properties or the stoichiometric ratio of the furosemide- or bumetanide-sensitive Na and K fluxes (Dunham et al., 1980).

Even though a pharmacological approach has been very successful in defining the modes of operation of the ouabain-sensitive Na pump, this does not seem to work for the cotransport systems, because the loop diuretics are not specific inhibitors of these systems. The specificity of ouabain and other cardiac glycosides as inhibitors of the Na pump has allowed studies of the interactions between Na and K ions transported by this transport system. In several studies, it has been shown that ouabain-sensitive (OS) Na and K transport can occur in at least five different modes: Na_i/K_o exchange, Na_i/Na_o exchange, K_i/K_o exchange, K_i/Na_o exchange, and uncoupled Na efflux (Glynn and Karlish, 1975). The characterization of these OS modes of transport has been an useful tool in the understanding of the partial chemical reactions catalyzed by the Na pump.

In order to gain further insight into the interactions between Na, K, and Cl, which can be ascribed to transport through an Na/K/Cl cotransport system, we investigated the properties of the ouabain-resistant (OR) Na and K fluxes in human red cells, studying the effect of furosemide, bumetanide, and Cl removal. Our interest in human red cells also derived from recent findings of alterations of furosemide-sensitive (FS) Na and K effluxes in the red cells of patients with essential hypertension (Garay et al., 1983; Canessa et al., 1984*b*). Genetic factors seem to play an important role in the FS Na and K efflux, as suggested by the family aggregation of these abnormalities (Cusi et al., 1981) and by the high heritability coefficient of the maximal rate of the FS Na and K fluxes found in human red cells of monozygous and dizygous twins (Lewitter and Canessa, 1984). It is very likely, therefore, that the interindividual variance in these ion fluxes derives not only from different alleles determining the number of transport sites but also from a different assembly of subunits.

The present study was designed to investigate the interactions between Na, K,

and Cl ions, which can be ascribed to a cotransport system and provide information about different modes of transport and their contribution to the overall transport reaction. The definition of the similarities and differences in these interactions will be helpful in distinguishing between the three types of cotransport (Na/Cl, K/Cl, and Na/K/Cl).

The present experiments describe several operational modes of the FS Na and K fluxes in human red blood cells. We report six modes of transport of Na and K that can be inhibited by furosemide. We provide evidence for the outward and inward cotransport of Na and K, exchange of internal K with external K, exchange of internal Na for external Na, uncoupled K efflux, and uncoupled Na efflux. We propose, from the analysis of these data, a reaction scheme for the FS Na and K fluxes. Some, but not all, of these modes reflect *cis* and *trans* interactions between Na, K, and Cl ions through an Na/K/Cl cotransport system.

Preliminary results of this work have been reported (Brugnara et al., 1983; Canessa et al., 1984*a*).

MATERIALS AND METHODS

Chemicals

KCl, NaCl, and $MgCl_2$ were purchased from Mallinckrodt, Inc., St. Louis, MO. Tris(hydroxymethyl)aminomethane (Tris), albumin (bovine, fraction V), 3-(*N*-morpholino)propanesulfonic acid (MOPS), and ouabain were purchased from Sigma Chemical Co., St. Louis, MO. *N*-Methyl-D-glucamine was purchased from Aldrich Chemical Co., Inc., Milwaukee, WI. Choline and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) were purchased from Calbiochem-Behring Corp., La Jolla, CA. Radioisotopes (^{22}Na, ^{42}K, and ^{86}Rb) were purchased from New England Nuclear, Boston, MA. Furosemide was a gift of Hoechst-Russel Pharmaceuticals, Inc., Somerville, NJ. Bumetanide (Laboratoire Leo, Vernouillet, France) was a gift from Dr. G. Dagher (INSERM, Hôpital Necker, Paris, France). Nystatin was from E.R. Squibb & Sons, Inc., Princeton, NJ.

All the solutions were prepared using double-distilled water. The osmolarity of the washing solutions and of the incubation media was adjusted to 295–305 mosmol.

Preparation of Red Cells

Blood was drawn from three males, ages 26, 27, and 32. One of the individuals (A.K.) was born to a hypertensive father and a normotensive mother in the United States. The other two (C.B. and D.C.) were born to normotensive parents in Italy. Most of the experiments were repeated in the three subjects and some were repeated in two subjects as indicated in the tables and figures. The blood was drawn into heparin, centrifuged in a Sorvall refrigerated centrifuge (RC 5B; Dupont Instruments, Newton, CT) for 10 min at 3,000 *g* for red cell separation, washed three times with isotonic choline washing solution (CWS) containing 150 mM choline Cl, 1 mM $MgCl_2$, and 10 mM Tris-MOPS, pH 7.4 at 4°C, and suspended in an approximately equal volume of CWS. From this suspension of fresh cells, determinations of hematocrit, cell Na (1:50 dilution in 0.02% Acationox; American Scientific Products, Boston, MA), cell K (1:500 dilution), and hemoglobin (optical density at 540 nm) were carried out.

Nystatin Loading Procedure

We have modified the original procedure developed by Cass and Dalmark (1973) by using substantially lower amounts of nystatin per milliliter of red cells (200 μg/ml) to facilitate the removal of the ionophore.

Cell titration. 6 ml of the choline-washed cells were centrifuged and suspended in 30 ml of the nystatin loading solution (NLS) at room temperature. The NLS contained 140 mM NaCl or KCl, in different ratios according to the desired Na and K content, and 34 mM sucrose. The cell suspension was brought to pH to 6.8 at 22°C ($Cl_i/Cl_o = 1$) by titration with 40% CO_2.

Incorporation of nystatin. The cell suspension was centrifuged and washed with cold NLS. 1 ml of packed cells was then added to 5 ml of cold NLS containing 40 μg/ml of nystatin. The nystatin was dissolved in dimethyl sulfoxide (10 mg in 2.5 ml). The cell suspension was then incubated at 4°C for 20 min. When cells with low Na or K content were needed, the supernatant was removed and the cells were incubated again for 20 min in a larger volume of cold loading solution without nystatin. Because of the higher partition coefficient of nystatin into the cell membrane at 4°C than at room temperature (Cass et al., 1970), enough antibiotic remains in the membrane to allow equilibration of the cation content of the cell with the incubation medium.

For the radioactive cation loading, after the first incubation at 4°C, the cells were suspended at 60–70% hematocrit in the same cold loading solution (without nystatin), which contained 10 μCi ^{22}Na, 150 μCi ^{86}Rb, or 400 μCi ^{42}K/3 ml of red cells. A 20-min incubation at 4°C with adequate shaking equilibrated the specific activities of medium and cells.

Removal of nystatin. The cold suspension was centrifuged for 5 min at 5,000 *g* and the cells were suspended for 10 min at 10% hematocrit in nystatin washing solution (NWS). The NWS had the same composition as the NLS, with the addition of 1 mM $KHPO_4$ buffer, pH 7.4, 10 mM glucose, and 0.1% albumin. The cells was then washed three times with NWS at room temperature, and centrifuged for 2 min at 5,000 *g* in a Sorvall centrifuge.

Removal of external cations. External cations were removed with five washes in CWS. After the final wash, the cells were suspended in equal volumes of CWS for determination of hematocrit, hemoglobin, and cell electrolytes as previously described.

Reproducibility of the nystatin loading. In every batch of cells loaded with the nystatin procedure, the cell volume was checked by measuring the hemoglobin per liter cell in the fresh and loaded cells. The initial cell volume was 99% ± 2 recovered. The rate constant of the furosemide-resistant (FR) Na and K fluxes was measured in fresh and nystatin-treated cells as a parameter of preservation of the normal passive permeability. In order to exclude nystatin effects, experiments done in fresh cells were repeated loading the cells to the same Na and K content as in the nystatin loading procedure. Similar results were obtained in fresh and nystatin-loaded cells. Control experiments for ^{22}Na efflux were performed in nystatin-treated cells and in cells incubated for 3 h at 37°C in a solution containing (mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $KHPO_4$ buffer, pH 7.4, 10 glucose, 10 Tris-MOPS, pH 7.4 at 37°C, and 10 μCi ^{22}Na/ml of medium. Similar results were obtained. ATP measurements before and after nystatin loading gave similar values.

Protocol for Measurement of Radioactive Unidirectional Efflux

The cells were washed six times with CWS, centrifuged for 2 min at 5,000 *g*, and suspended in an approximately equal volume for determination of hematocrit and Na and K content as previously described. 1 ml of cell suspension was added to 10 ml of medium. The media contained 150 mM choline Cl (choline media) or 130 mM NaCl and 20 mM choline Cl (Na media). The choline plus K media contained 100 mM choline Cl and 0–50 mM KCl, with corresponding adjustments in the choline concentration to keep the sum of K plus choline equal to 150 mM. The Na plus K media contained 130 mM NaCl and 0–20 mM KCl, with corresponding adjustments of the choline concentration to

keep the sum of K plus choline equal to 20 mM. All media contained (mM): 10 Tris-MOPS, pH 7.4 at 37°C, 10 glucose, and 0.1 ouabain.

The cold flux suspension (11 ml) was vortexed and 1.1 ml was distributed with automatic pipettes into five previously chilled, 5-ml plastic tubes. Afterwards, 6 μl of a neutralized solution of furosemide (33 mg plus 100 μl of 1 M Tris-OH) was added to the 5.5 ml of the remaining suspension of red cells, vortexed, and distributed into another five tubes as previously done with the medium containing only ouabain. After capping, two of the tubes were incubated for 5 min and three were incubated for 65 min at 37°C for each condition, with and without furosemide. The reaction was stopped by cooling for 2 min in an ice bath and subsequently centrifuging at 1,000 *g* for 5 min. 0.8 ml of supernatant was removed and counted with at least 0.5% precision in a gamma counter (Auto-Gamma 500, Packard Instruments, Lynn, MA). Five aliquots of 1 ml of a 1:50 dilution of the initial cell suspension in CWS were counted for determination of the initial specific activity (SA).

The efflux in millimoles per liter cells per hour was calculated from:

$$\text{efflux} = 1/\text{cell SA} \times 1/V_s \times 1{,}000 \times F \times \text{cpm/h},$$

where cell SA is counts per minute per millimole, F is milliliters flux medium per milliliter of red cells, V_s is the volume (in liters) of supernatant counted, and cpm/h is the counts per minute in the supernatant of the samples taken after 65 min incubation minus the counts per minute in the supernatant of the sample taken after 5 min incubation. The standard error of the slope of counts per minute vs. time was used to calculate the standard error of the fluxes.

Protocol for Measurement of Unidirectional Radioactive Influx

10 ml of medium was chilled and mixed with 10 μCi of ^{22}Na or 30 μCi of ^{86}Rb or 200 μCi of ^{42}K. The radioactivity in five aliquots of 20 μl of medium was measured for determination of the initial specific activity. 0.6–0.8 ml of packed red cells was added to the 10 ml of medium. The influx medium was then processed as previously described for the efflux. The supernatant of the samples, collected after 5 and 25 (or 65) min incubation at 37°C, was discarded and the cells were washed three times with 4 ml of cold Na washing solution containing (mM): 150 NaCl, 10 Tris-MOPS, pH 7.4 at 4°C, and 0.1 ouabain. Afterwards, the cell pellet was lysed with 1 ml of double-distilled water containing 0.02% Acationox. The tubes were vortexed and spun for 20 min at 3,000 *g*. Aliquots of 50 μl were diluted 50 times with an automatic dilutor for hemoglobin determinations by measuring the optical density at 540 nm. Aliquots of 0.8 ml were counted in a gamma counter as described before.

The influx in millimoles per liter cells per hour was calculated from:

$$\text{influx} = \frac{\text{cpm/liter red cells (65 min} - \text{5 min incubation)}}{\text{ISA}^{(\text{cpm/mmol})}},$$

where cpm/liter of red cells is the counts per minute in the lysate × $1/Vl$ × 100/Hct. Vl is the volume (liters) of lysate counted and Hct is the amount of cells (percent) in the lysate. The amount of red cells present in the lysate was calculated using the hematocrit and concentrations of hemoglobin of the initial cell suspension and the concentration of hemoglobin determined in the lysate. ISA is the initial specific activity of the medium in counts per minute per millimole. The standard error of the influx was calculated from the standard error of the slope of the regression of time vs. intracellular Na or K content.

In preliminary experiments, we determined the time course of OR and FS ^{22}Na influx and efflux in fresh cells. Both were found to be linear up to 75 min. However, it should

be noted that in cells with low Na content, the cell Na changes significantly with these incubation times. Hence, for cells containing <5 mmol Na/liter cell, shorter incubation times should be used to study the unidirectional Na fluxes.

Cl Replacement and DIDS Treatment

Nystatin-loaded cells were separated after the fourth warm wash into two fractions, control and Cl free. Cl was removed by two washes, incubation for 30 min, and two other washes in (mM): 150 glucamine nitrate, 1 $MgNO_3$, and 10 Tris-MOPS, pH 7.4 at 4°C. The efflux medium contained (mM): 150 glucamine, 1 Mg [Cl_2 or $(NO_3)_2$], 10 Tris-MOPS, pH 7.4 at 37°C, 10 glucose, and 0.1 ouabain.

The DIDS treatment was performed as described by Funder et al. (1978).

Cation Determination by Atomic Absorption

Red Cells. Na and K contents of the cells were measured in dilutions in double-distilled water containing 0.02% Acationox. The Na and K contents were determined in an atomic absorption spectrophotometer (model 5000, Perkin-Elmer Corp., Norwalk, CT) using standards in water.

Media. The Na and K concentrations in the flux supernatants of the choline media were measured using standards of Na and K in 150 mM choline. The K efflux in Na medium was measured using standards of K in 130 mM NaCl.

RESULTS

FS Outward Cotransport of Na and K

Activation of the FS Na and K effluxes by internal K and Na. Outward Na/K cotransport was previously investigated measuring FS Na and K effluxes into 75 mM $MgCl_2$ medium by increasing intracellular Na with the 2,5-chloro-*p*-mercuribenzene sulfonate (PCMBS)-loading procedure (Garay et al., 1981). In the present experiments, two different conditions were used: the cells were loaded by the nystatin procedure, and the efflux medium contained lower $MgCl_2$ concentrations (1 mM) in order to avoid the inhibition of the outward Na/K cotransport by external Mg (Ellory et al., 1980, 1983; Bize et al., 1982). In this loading procedure, we varied internal Na and K simultaneously. We did not use choline loading to keep the concentration of one ion constant, because the higher concentration of nystatin (100 μg/ml) required for choline loading markedly increased the FR Na and K fluxes. Thus, the measurement of kinetic parameters ($K_{0.5}$ and V_{max}) under the conditions we used has only a comparative and not an absolute value.

The activation curve of FS Na efflux by internal Na (Na_i) and K (K_i) is shown in Fig. 1. The apparent K_m ($K_{0.5}$) values for Na_i and K_i were calculated from the Eadie plot. In the three subjects studied, the Na_i concentration, which half-stimulated the FS Na efflux, was 14 mmol/liter cell in high-K cells. The $K_{0.5}$ for K_i to half-stimulate the FS Na efflux (4 mmol/liter cell) in cells with high Na content was significantly lower than the $K_{0.5}$ for Na_i to stimulate the FS Na efflux in high-K cells (Table I). It seems, therefore, that to half-activate the FS Na efflux, less K_i is required in high-Na cells than Na_i in high-K cells.

The dependence of the FS K efflux on Na_i and K_i concentrations is shown in Fig. 2. At high cellular Na, the FS K efflux was half-activated by 13 mmol K/

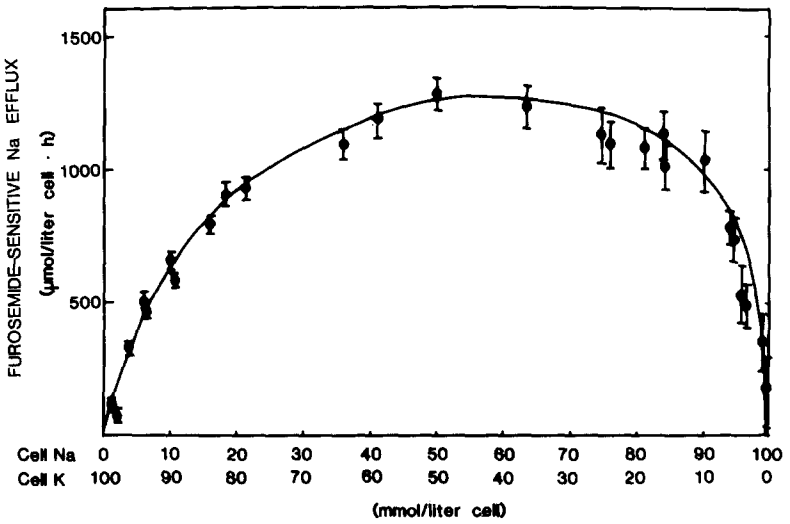

FIGURE 1. FS Na efflux (± SE) into Na- and K-free medium, as a function of cellular Na and K contents. Cell Na and K were reciprocally varied using the nystatin technique. The efflux medium contained (mM): 150 choline chloride, 1 $MgCl_2$, 10 Tris-MOPS, pH 7.4 at 37°C, 10 glucose, 0.1 ouabain, with and without 1 furosemide. The hematocrit of the efflux media was 2%. The incubation times were 5, 35, and 65 min. (Subject C.B. Similar results were obtained in the other two subjects.)

liter cell, whereas at high cellular K, the FS K efflux was half-activated by 8 mmol Na/liter cell (Table I). Therefore, less Na_i was required to half-activate the FS K efflux in high-K cells than K_i to half-activate FS K efflux in high-Na cells.

In choline media, there was no FS Na efflux from cells containing only Na and there was no FS K efflux from cells containing only K (Figs. 1 and 2).

TABLE I

Kinetic Parameters of the Activation Curve by Internal Na and K of the FS Na and K Effluxes into Choline Medium

	Activation by internal Na ($K_{0.5}$)		Activation by internal K ($K_{0.5}$)	
Subject	Na efflux	K efflux	Na efflux	K efflux
	mmol/liter cell		*mmol/liter cell*	
A.K.	13.4±1.2	6.4±1.6	3.0±1.4	9.0±1.4
C.B.	15.0±1.2	7.7±1.3	4.0±1.6	17.0±1.3
D.C.	13.4±2.8	10.1±1.5	4.5±1.8	14.0±1.1
Mean ± SD	13.9±0.8	8.1±1.5	3.8±0.6	13.3±3.3

The internal Na and K concentrations were reciprocally varied with the nystatin loading procedure. The cells were incubated in an efflux medium containing (mM): 150 choline Cl, 1 $MgCl_2$, 10 Tris-MOPS, pH 7.4 at 37°C, 10 glucose, and 0.1 ouabain, with and without 1 furosemide. The hematocrit of the efflux medium was 2%. The efflux times were 5, 35, and 65 min. The $K_{0.5}$ ± SEM was calculated from the Eadie plot.

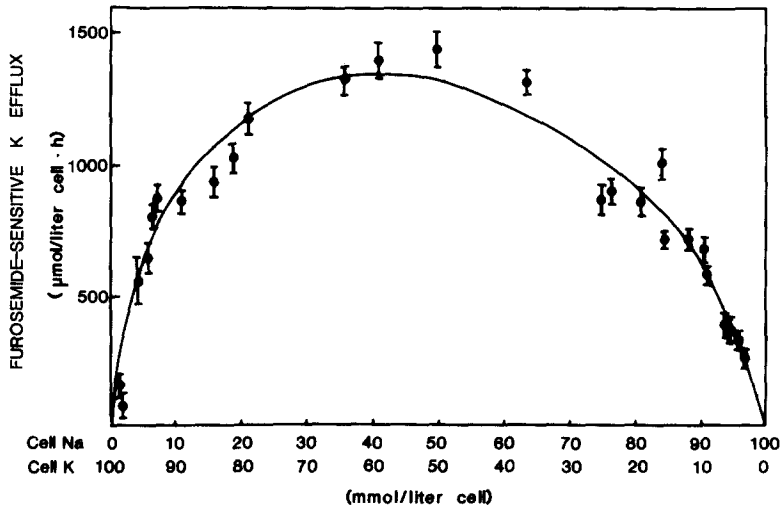

FIGURE 2. FS K efflux (± SE) into Na- and K-free medium as a function of cellular Na and K content. Experimental conditions were the same as in Fig. 1. (Subject C.B. Similar results were obtained in the other two subjects.)

FR Na and K effluxes into Na- and K-free media. Fig. 3 shows the dependence on internal Na and K of the OR and ouabain- and furosemide-resistant (OR-FR) Na effluxes into choline medium containing 1 mM $MgCl_2$. It can be seen that both fluxes increased progressively, with increasing internal Na. However, the

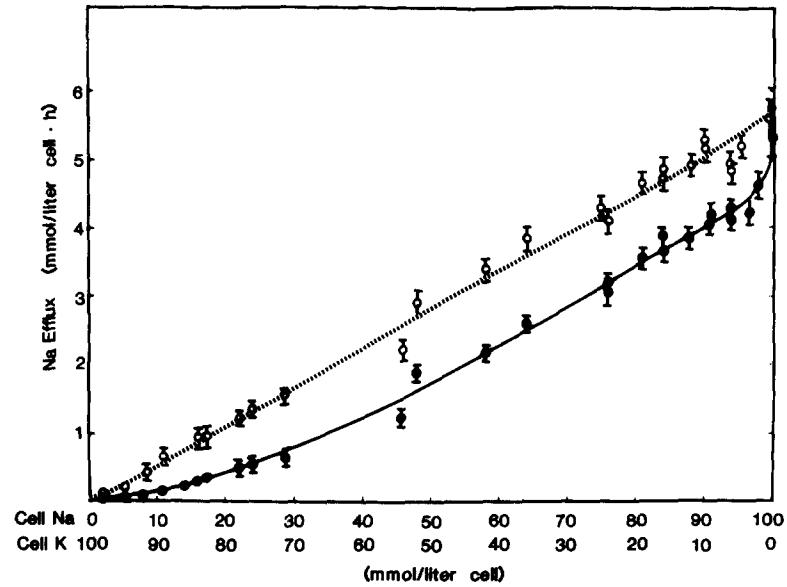

FIGURE 3. OR (○) and FR (●) Na efflux (± SE) into Na- and K-free media as a function of cellular Na and K contents. Experimental conditions were the same as in Fig. 1. (Subject C.B. Similar results were obtained in the other two subjects.)

curve relating the OR Na efflux to Na_i was linear over the entire range (rate constant, ~0.05 h^{-1}), whereas the dependence of the OR-FR Na efflux on Na_i was clearly not linear. The slope increased with increasing Na_i in the region Na_i = 0–30 mmol/liter cell, remained constant at a value of ~0.04 h^{-1} in the region Na_i = 30–90 mmol/liter cell, and rose again in the region Na_i = 90–100 mmol/liter cell. The reason for this nonlinearity of the OR-FR Na efflux as a function of Na_i is not clear. The fall in the rate constant with decreasing Na_i at Na_i = 100–90 mmol/liter cell could be due to inhibition by K_i, but the fall in the rate constant with decreasing Na_i at Na_i = 30–0 mmol/liter cell is unlikely to stem from this source. Further experiments with internal cations other than K will be

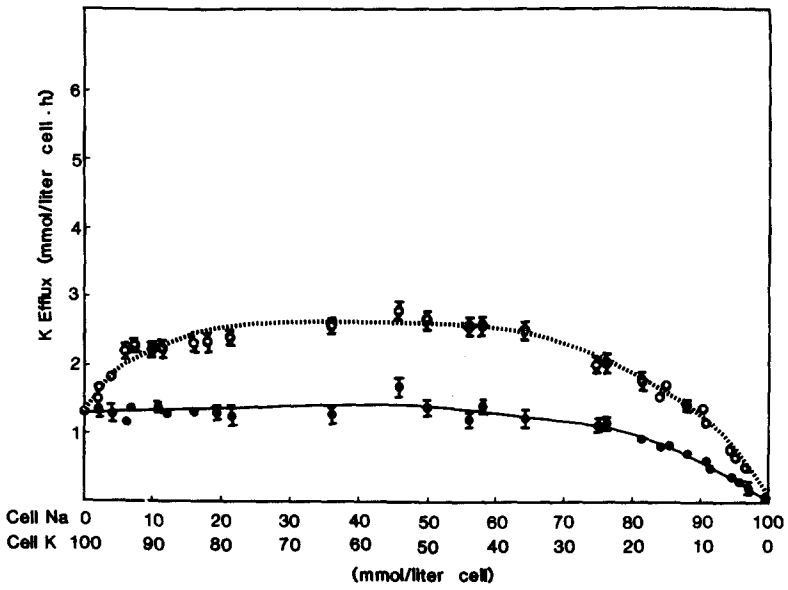

FIGURE 4. OR (○) and FR (●) K efflux (± SE) into Na- and K-free media as a function of cellular Na and K contents. Experimental conditions were the same as in Fig. 1. (Subject C.B. Similar results were obtained in the other two subjects.)

necessary to resolve this point. Meanwhile, it is interesting to note that the bell-shaped curve of the FS Na efflux shown in Fig. 1 (the difference between the two curves shown in Fig. 3) is not the result of a nonlinear curve in the absence of furosemide and a linear curve in the presence of the inhibitor, as might be expected if the OR-FR Na efflux were due to electrodiffusion, but rather to a curve that was more linear in the absence than in the presence of furosemide.

Fig. 4 shows the dependence on Na_i and K_i of the OR and OR-FR K effluxes. It can be seen that the OR K efflux was *cis*-stimulated by internal Na. The OR-FR K efflux was constant over a range from 100 to 40 mmol/liter cell of K_i. The increase in cell Na did not seem to inhibit the OR-FR K efflux. On the contrary, the rate constant of the OR-FR K efflux increased with cell Na from 0.015 h^{-1} (Na_i = 0–25 mmol/liter cell) to 0.032 h^{-1} (Na_i = 50 mmol/liter cell) and to 0.050 h^{-1} in high-Na, low-K cells.

Garay et al. (1981) showed that the rate constant for the OR-FR effluxes into

media with high $MgCl_2$ concentrations (75 mM) was 0.019 h^{-1} for Na and 0.014 h^{-1} for K, and the Na and K effluxes were a linear function of the internal Na or K concentration. The present results indicate that in the presence of physiological Mg concentrations in the incubation medium, the OR-FR leakage pathways for Na and K are different and they are not linear functions of cell Na and K. Therefore, they should not be considered to be only diffusional leak pathways.

cis stimulation, Cl dependence, and bumetanide sensitivity of the OR Na and K effluxes in Na- and K-free media. It has been reported that bumetanide inhibits the OR Na and K fluxes at lower concentrations than does furosemide (Palfrey and Rao, 1983). Several studies have shown that a sizable component of the OR Na and K fluxes is Cl dependent (Chipperfield, 1981; Dunham et al., 1980). Therefore, we have also studied the effects of bumetanide (0.01 mM), Cl replacement by glucamine nitrate, and the *cis* stimulation of the OR Na and K effluxes. *cis*-stimulated K efflux was assessed by comparing the OR K efflux from cells containing only K with that of cells containing increasing amounts of Na. *cis*-stimulated Na efflux was studied by comparing the OR Na efflux from cells containing only Na with that of cells containing increasing amounts of K.

The maximal rates of FS, bumetanide-sensitive, and Na_i-stimulated K effluxes all had similar values (1,400 μmol/liter cell·h), but the Cl-dependent K efflux was 30% lower than the FS K efflux. An FS component (30%) of the K efflux was present in the nitrate media. However, internal Na did not *cis*-stimulate the OR K efflux into nitrate media (data not shown).

The maximal rates of FS and bumetanide-sensitive Na effluxes were equal, but the Cl-dependent Na efflux was lower than the FS Na efflux and there was an FS Na efflux into nitrate media. We also found that there was no stimulation by internal K of the OR Na efflux into Cl media. We will show later that the rate constant of FR Na efflux from cells containing only Na is significantly higher than that of the FR K efflux from cells containing only K, and that the absence of a K_i-stimulated Na efflux is caused by the marked inhibition of the FR Na efflux by internal K.

FS Uncoupled K Efflux

There was no FS K efflux into choline medium (1 mM $MgCl_2$) from cells containing only K. However, when these cells were incubated in the presence of increasing amounts of K_o, the FS K efflux increased to 500 μmol/liter cell·h (Fig. 5). The half-maximal stimulation of the FS K efflux was reached at K_o = 5 mM. Fig. 5 also shows the measurement of FS K influx in the same experimental conditions. It can be seen that external K did not stimulate FS K influx. In this particular experiment, the rate constant of the OR-FR K influx was 0.010 h^{-1}. This value was similar to the rate constant of the OR-FR K influx in cells containing K and different amounts of Na. FS K efflux without inward transport of K cannot take place through a K_i/K_o exchange pathway. We propose to name this component of the FS K efflux the "uncoupled K efflux," because it takes place in the absence of internal Na and in the presence of K_o, without simultaneous inward K transport. Another interesting property of the uncoupled K efflux was that it was stimulated by DIDS treatment or by replacement of Cl by

nitrate. These results suggest that this flux component may not be a transport mode of the Na/K/Cl cotransport.

FS K_i/K_o Exchange

Internal Na stimulates the OR and FS K influx. The observation that a sizable component of K influx in the absence of external Na is furosemide sensitive has been interpreted as evidence for the presence in human red cells of a KCl cotransport system similar to the one present in LK sheep red cells (Dunham et al., 1980; Dunham and Ellory, 1981; Ellory et al., 1982). The following experiments indicate that the Na_o-independent K influx is a K_i/K_o exchange pathway of the Na/K cotransport system.

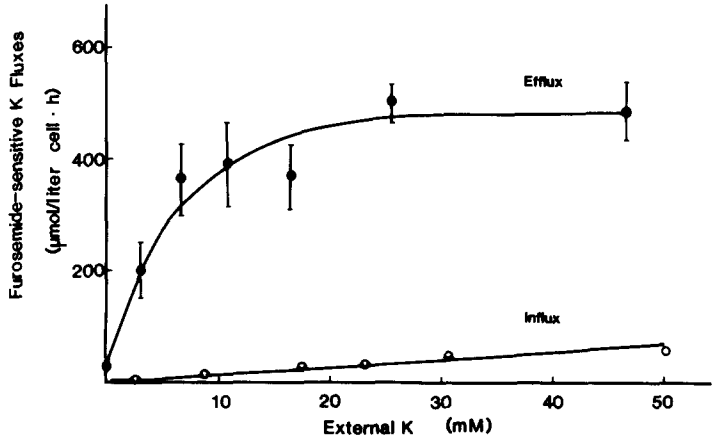

FIGURE 5. FS K efflux and influx (± SE) as a function of external K concentration in Na-free cells (internal Na and K = 0.5 and 90 mmol/liter cell, respectively). The fluxes were measured in media containing (mM): 150–100 choline chloride, 0–50 KCl, 1 $MgCl_2$, 10 Tris-MOPS, pH 7.4 at 37°C, 10 glucose, 0.1 ouabain, with and without 1 furosemide. The hematocrit of the media was 5% for the influx and 2% for the efflux. ^{42}K was used as tracer. This is one of two experiments. (Subject C.B. Similar results were obtained in another subject.)

Fig. 6*A* shows the OR K influx as a function of external K in Na-free medium at different intracellular Na concentrations. It can be seen that internal Na stimulated the OR K influx. As shown in Fig. 6*B*, the stimulation produced by internal Na involved an FS component of the K influx.

Table II shows the kinetic parameters of the FS K influx for the three subjects studied. The $K_{0.5}$ for external K ranged from 11 to 23 mM and was not affected by the internal Na concentration. The V_{max} of the FS K influx increased with the elevation of internal Na. The stimulatory effect of internal Na on the FS K influx was half-maximal at 5 mmol/liter cell of Na.

As can be seen in Fig. 6, in Na_i-free cells, the OR and FR K influxes were a linear function of external K. In cells containing Na, the FS K influx was a saturable function of the external K concentration. These data indicate that internal Na had a stimulatory role on the Na_o-independent K influx.

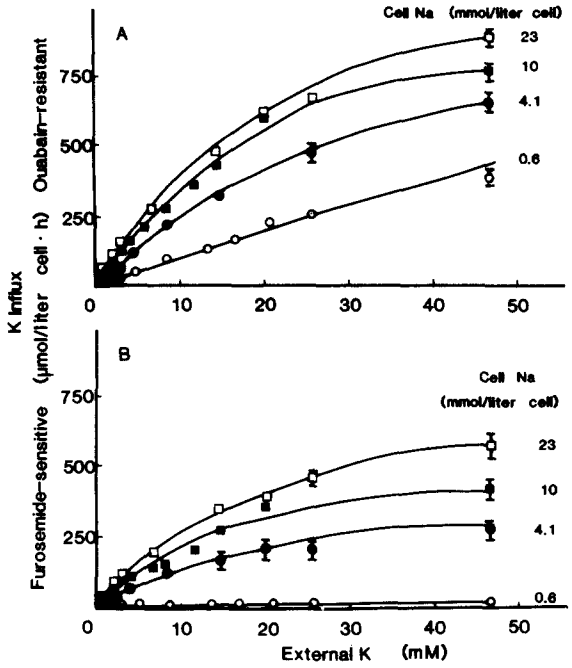

FIGURE 6. In *A*, the OR K influxes (± SE) are plotted as a function of external K, into cells containing 0.6, 4.1, 10, and 23 mmol/liter cells of Na. In *B*, the FS components of the K influxes (± SE) of the same experiments are plotted. The influxes were measured in media containing (mM): 150–100 choline chloride, 0–50 KCl, 1 $MgCl_2$, 10 Tris-MOPS, pH 7.4 at 37°C, 10 glucose, 0.1 ouabain, with and without 1 furosemide. The hematocrit of the influx media was 5%. ^{42}K was used as tracer. (Subject A.K. Similar results were obtained in the other two subjects.)

TABLE II

FS K Influx from Choline Medium (K_i/K_o Exchange) at Different Cellular Na

	Intracellular		FS K influx	
Subject	Na	K	K_m for K_o	V_{max}
	mmol/liter cell		*mM*	*μmol/liter cell·h*
A.K.	4.5	88	23±2.4	400±35
	11.5	85	11±5	380±65
	24.0	70	20±0.5	820±15
C.B.	3.9	85	21±1.5	380±20
	6.5	87	15±1.5	350±20
	7.5*	87	15±3.5	380±35
	24.5	71	21±2	705±50
	22.0*	76	19±2.2	850±60
D.C.	11.0	93	16±2.7	290±20
			Mean ± SD: 17.8±3.9	

The internal Na concentration was varied with the nystatin technique. The influx media contained (mM): 150–100 choline Cl, 0–50 KCl, 1 $MgCl_2$, 10 Tris-MOPS, pH 7.4 at 37°C, 10 glucose, and 0.1 ouabain, with and without 1 furosemide. The hematocrit of the influx media was 4%. ^{86}Rb was used as a tracer. *^{42}K was used as a tracer. The $K_{0.5}$ and V_{max} (± SEM) values were calculated from the Eadie plot.

Internal K stimulates the OR and FS K influx. If the Na_o-independent K influx takes place through an FS K_i/K_o exchange pathway of the Na/K cotransport, it should be stimulated not only by internal Na but also by internal K. Table III shows that the OR and FS K influx was stimulated by internal K in cells with high Na content and that there was no FS K influx into cells containing only Na. These results suggest that the FS K_i/K_o exchange is a mode of operation of the FS Na/K cotransport, since it requires the presence of both Na and K on the inside of the membrane.

External K stimulates the OR and FS K efflux. If the Na_o-independent K influx takes place through an FS K_i/K_o exchange pathway of the FS Na/K cotransport, external K should stimulate K efflux from cells containing Na and K. The effect of external K on K efflux was investigated in cells containing 7

TABLE III

Stimulation by Internal Na and K of the OR and FS K Influx from Choline Medium

Intracellular		K influx		
Na	K	OR	FR	FS
mmol/liter cell		*μmol/liter cell·h*		
90	1.5	445±15	460±25	—
87	8.5	540±20	470±6	70±20
76	19.0	635±40	420±10	215±40
1	91	245±5	220±5	25±5
4	85	350±10	170±10	180±10
7	87	410±10	210±5	200±10
24	71	550±10	170±5	380±10

The internal Na and K concentrations were varied with the nystatin technique. The influx media contained (mM): 130 choline Cl, 20 KCl, 1 $MgCl_2$, 10 Tris-MOPS, pH 7.4 at 37°C, 10 glucose, and 0.1 ouabain, with and without 1 furosemide. The hematocrit of the influx media was 4%. ^{86}Rb was used as tracer. Subject C.B. (Fluxes ± SE.)

and 22 mmol/liter cells of Na. Fig. 7 shows the effect of increasing external K (0–50 mM) on the FS K efflux into choline medium from cells with 22 mmol/liter cells of Na and 73 mmol/liter cells of K. External K stimulated the OR K efflux. This K_o-stimulated K efflux was furosemide sensitive. We show in another section of this paper that external K inhibits FS Na efflux. In order to estimate the component of the FS K efflux transported by cotransport of Na and K, we assumed that the outward transport of Na is coupled to K with a similar ratio in the presence and in the absence of external K. In Fig. 7, we have subtracted from the FS K efflux the calculated cotransport of K with Na. We named the remaining component of the FS K efflux the "extra K efflux." It can be seen in Fig. 7 that the extra K efflux was equal to the FS K influx, in agreement with a 1:1 K_i/K_o exchange pathway of the FS Na/K cotransport.

Other properties of the FS K_i/K_o exchange pathway. We compared in fresh red cells of one subject the magnitudes of FS, bumetanide-sensitive, and Cl-dependent K influx at 20 mM external K into fresh cells. These components were 270,

210, and 170 μmol/liter cell·h, respectively. This would suggest that the K_i/K_o exchange is at least 60% Cl dependent. However, we observed an FS component of the K influx (70 μmol/liter cell·h) from glucamine nitrate medium. This would indicate that, at high concentrations, nitrate may be able to replace in part Cl in the FS K_i/K_o exchange pathway.

FS Inward Cotransport of Na and K

External Na stimulates the FS K influx. The inward Na/K cotransport can be recognized by the *cis* stimulation of the OR K influx by external Na, as previously described in human red cells by Wiley and Cooper (1974), and in

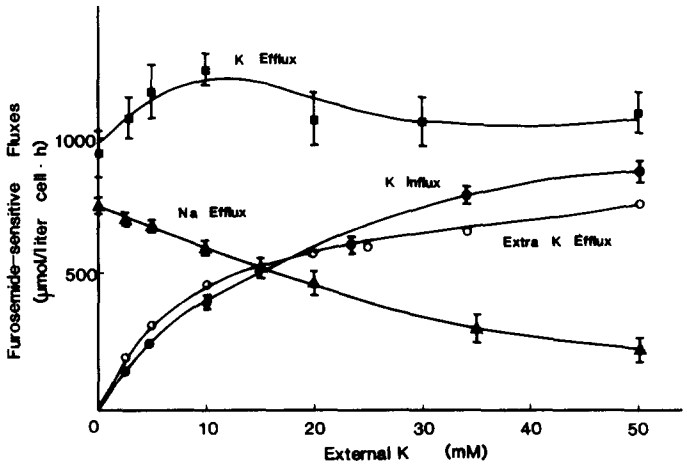

FIGURE 7. FS K and Na effluxes and K influx (± SE) as a function of external K. The cell Na content was 22 mmol/liter cell. The K efflux by cotransport was calculated by multiplying the FS Na efflux, in the presence of external K, by the stoichiometric ratio of FS K to Na effluxes in the absence of external K. The extra K efflux was calculated by subtracting the K efflux by Na/K cotransport from the FS K efflux. The media contained (mM): 150–100 choline chloride, 0–50 KCl, 1 $MgCl_2$, 10 Tris-MOPS, pH 7.4 at 37°C, 10 glucose, 0.1 ouabain, with and without 1 furosemide. (Subject C.B. Similar results were obtained in another subject.)

duck red cells by Schmidt and McManus (1977*a*, *b*). Fig. 8*A* shows a plot of the FS and Na_o-stimulated K influx into cells containing only K ($Na_i < 0.5$ mmol/liter cells). The Na_o-stimulated component of the OR K influx estimates the inward coupled transport of Na and K. In Na-free cells, the two components of the OR K influx (FS and Na_o-stimulated) were equal (the difference of ±50 μmol/liter cell·h between the two fluxes was within the limits of experimental error). In the same cells, the FS K_i/K_o exchange was negligible. Fig. 8*B* shows the measurement of FS and Na_o-stimulated K influx into cells containing 22 mmol/liter cells of Na and 73 mmol/liter cells of K. It can be seen that when the cells contained Na, the absolute magnitude of the FS K influx was two to three times higher as compared with Fig. 8*A*, and the Na_o-stimulated component was significantly lower than the total FS K influx. The difference between the two

components can be accounted for by the presence of the K_i/K_o exchange pathway, which is stimulated by internal Na and can be estimated by measuring the FS K influx from Na-free medium. This pathway seems to operate in parallel with the outward and inward cotransport of Na and K. Fig. 9*A* shows the FS K influx from Na-containing medium as a function of internal Na and external K. The

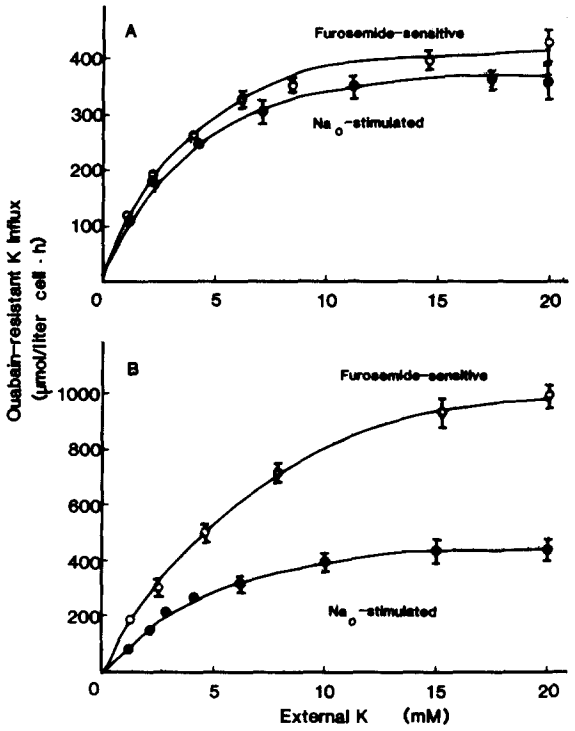

FIGURE 8. In *A* and *B*, the FS and Na_o-stimulated components (± SE) of the K influx from Na media are plotted. The Na_o-stimulated component was calculated as the difference between OR K influxes in Na and choline medium at any given external K concentration. In *A*, the cells contained 0.4 mmol Na/liter cell and 90 mmol K/liter cell. In *B*, the cells contained 22 mmol Na/liter cell and 76 mmol K/liter cell. The influxes were measured in media containing (mM): 130 choline chloride or 130 NaCl, 0–20 KCl, 20–0 choline chloride, 1 $MgCl_2$, 10 Tris-MOPS, pH 7.4 at 37°C, 10 glucose, 0.1 ouabain, with and without 1 furosemide. The hematocrit of the influx media was 5%. ^{42}K was used as tracer. (Subject C.B. Similar results were obtained in another subject.)

V_{max} of the FS K influx increased with internal Na. However, the Na_o-stimulated component was more or less similar in Na-free cells and in Na-containing cells (Fig. 9*B*). It seems, therefore, that the stimulation by internal Na of the FS K influx from Na medium does not reflect an activation of the inward coupled transport of Na and K, but of the K_i/K_o exchange pathway.

Other properties of the OR K influx. FS and bumetanide-sensitive K influx were measured by incubating fresh cells in a medium containing 130 mM Na

and 20 mM K. The bumetanide-sensitive K influx was 10% lower than the FS K influx. The Cl-dependent K influx (nitrate replacement) was 20% lower than the FS. The Na_o-stimulated component (inward cotransport of Na and K) was also significantly lower than the Cl-dependent K influx (data not shown). These differences can be accounted for by the presence in Na medium of a K_i/K_o exchange pathway, Cl dependent and Na_i stimulated, which operates in parallel with the inward cotransport of Na and K.

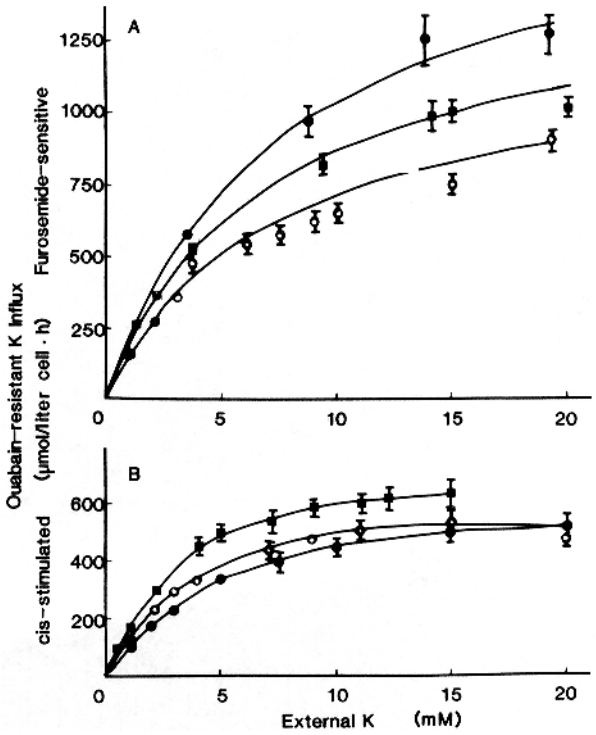

FIGURE 9. In *A*, the FS K influx (± SE) as a function of external K (130 mM NaCl medium) with different internal Na concentrations is plotted. In *B*, the *cis*-stimulated K influxes (Na_o-stimulated K influx) are plotted as a function of external K at different internal Na concentrations. In *A* and *B*, the intracellular Na concentration was varied with the nystatin loading procedure to 4.1 (○), 11 (■), and 23 (●) mmol/liter cell. Experimental conditions were similar to those of Fig. 8. (Subject A.K. Similar results were obtained in another subject.)

External K stimulates the FS Na influx. We showed in the previous paper (Brugnara et al., 1986) that external K stimulated the OR (*cis* stimulation) and FS Na influx ($K_{0.5} = 5$ mM). The FS Na influx was also stimulated when the cell Na content was increased. The ratio between the FS Na and K influxes was 2:3 when external K was higher than 10 mM.

FS Na_i/Na_o Exchange

It was shown in the previous paper (Brugnara et al., 1986) that in Na-free cells there was no FS Na influx in the absence of external K. Internal Na stimulated

($K_{0.5}$ = 5 mmol/liter cell) FS Na influx from a K-free medium. It is difficult to demonstrate that Na_o stimulated Na efflux, because external Na markedly inhibited FS Na (and K) efflux. However, assuming that FS Na influx in the absence of external K occurs by a 1:1 Na_i/Na_o exchange, external Na can simultaneously inhibit Na efflux by outward cotransport of Na and K and stimulate Na efflux by Na_i/Na_o exchange. Fig. 10 shows the mean value of FS Na influx from Na media, in the three subjects studied, at three different cell Na concentrations. It can be seen that the K_i/K_o exchange was four to six times larger than the Na_i/Na_o exchange.

The FS Na_i/Na_o exchange was stimulated by Cl removal (nitrate substitution) and this stimulation was inhibited by DIDS (120 μM). Bumetanide (0.01 mM)

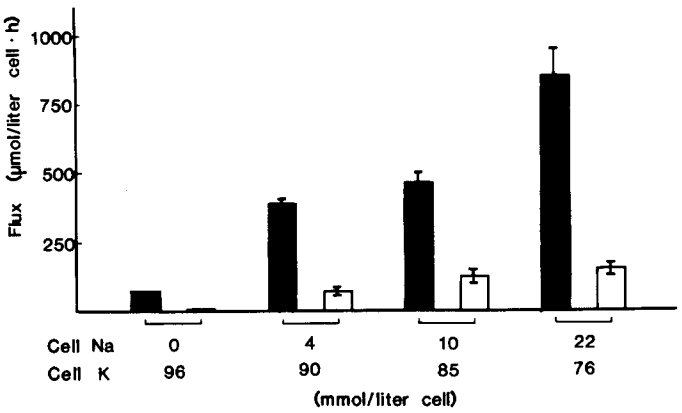

FIGURE 10. Maximal rates of FS Na_i/Na_o (□) and K_i/K_o (■) exchanges at different cellular Na contents. The mean values (± SEM) for the three subjects studied are plotted. The Na_i/Na_o exchange was measured as FS Na influx from a medium containing 140 mM NaCl. The K_i/K_o exchange was measured as FS K influx from a medium containing 140 mM KCl. All the media contained (mM): 1 $MgCl_2$, 10 Tris-MOPS, pH 7.4 at 37°C, 10 glucose, 0.1 ouabain, with and without 1 furosemide.

did not inhibit the OR Na influx from 130 mM NaCl medium. These results support the idea that the FS Na_i/Na_o exchange is not a pathway of the Na/K cotransport.

FS Uncoupled Na Efflux

Na efflux from cells containing only Na into choline medium is not inhibited by furosemide (Fig. 1). In the presence of external Na (130 mM), a sizable component of the OR Na efflux from cells containing only Na becomes furosemide sensitive (Table IV). However, the large increase in the permeability for Na in cells containing no K (Fig. 3) makes it difficult to show that increasing amounts of Na_o stimulated an FS uncoupled Na efflux. As with external K and FS K influx in cells containing only K (Fig. 5), external Na promoted a very small FS Na influx in cells containing only Na (150 μmol/liter cell·h). Therefore, the FS uncoupled Na efflux can be operationally defined as the net FS Na efflux from cells containing only Na into 130 mM NaCl medium (Table IV).

TABLE IV

FS Uncoupled Na Efflux in Human Red Cells

	Na efflux			Na influx			Net efflux
	OR	FR	FS	OR	FR	FS	FS
	mmol/liter cell·h			*mmol/liter cell·h*			
J.H.	2.92±0.1	2.50±0.09	0.42±0.14	2.93±0.07	2.79±0.07	0.15±0.10	0.270
N.E.	3.65±0.18	2.84±0.15	0.81±0.23	3.84±0.09	3.42±0.08	0.42±0.12	0.390
C.B.	3.82±0.12	3.42±0.06	0.40±0.14	2.68±0.06	2.63±0.02	0.05±0.07	0.350

The mean (± SD) red cell Na and K contents for the three subjects were 94.4 ± 1.3 and 0.3 ± 0.1 mmol/liter cell, respectively. ^{22}Na efflux and influx were measured in a medium containing (mM): 130 NaCl, 20 choline Cl, 1 $MgCl_2$, 10 Tris-MOPS, pH 7.4 at 37°C, 10 glucose, and 0.1 ouabain, with and without 1 mM furosemide. The hematocrit of the flux suspension was 2.5% for efflux and 6% for influx. The incubation times at 37°C were 5 and 25 min. (Fluxes ± SE.)

Variable Stoichiometry of FS Na and K Fluxes

Stoichiometry of FS Na and K effluxes into choline medium. As can be seen in Figs. 1 and 2, the stoichiometric ratio of FS Na and K effluxes can vary according to the relative ratio of Na and K inside the cell. Fig. 11 shows the relationship beween the ratio of cell Na/K contents and the ratio of FS Na/K effluxes in choline medium. When the internal Na/K ratio was $\gg 1$, the ratio of FS Na to K efflux was close to 2. When the internal Na/K ratio was <1, the ratio of FS Na to K efflux approached 0.5. These results differ from the 1:1 stoichiometry of FS effluxes into 75 mM $MgCl_2$ media reported by Garay et al. (1981). The origin of this discrepancy is not clear. Possibly, when the FS Na and K effluxes are maximally inhibited by $MgCl_2$, their small magnitude and larger error make it difficult to measure these changes in the stoichiometry of FS Na and K fluxes.

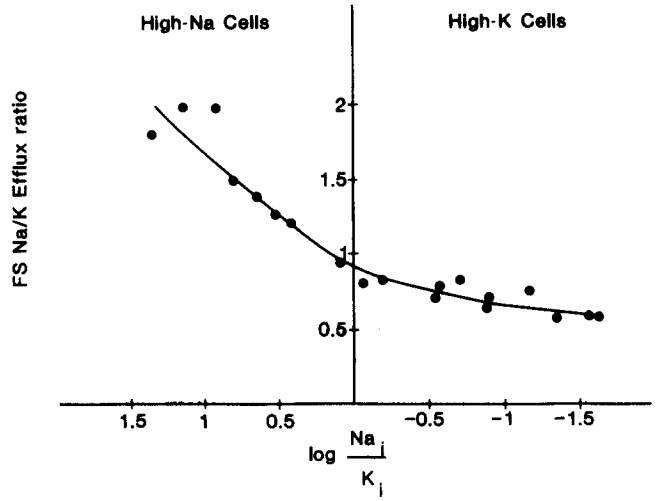

FIGURE 11. Stoichiometric ratio of FS Na and K effluxes as a function of the log of the ratio of intracellular Na and K contents. The results from the experiments shown in Figs. 1 and 2 were used for this plot.

The stoichiometric ratio of FS Na and K effluxes is changed by external K. Increasing external K produced an inhibition of the FS Na efflux into choline media. The Dixon plot of the K_o inhibition of FS Na efflux gave $K_{0.5}$ values of 20, 22, and 28 in the three subjects studied (Fig. 12). A plot of the reciprocal values of Na_i vs. the slope of the Dixon plot gave an apparent $K_{0.5}$ for Na_i of 13 mmol/liter cell. Thus, the $K_{0.5}$ for internal Na to activate FS Na efflux was the same in choline or choline plus K media (Table V). Therefore, external K is acting as a noncompetitive inhibitor of the FS Na efflux into choline media, since it reduced the V_{max} but not the $K_{0.5}$ for Na_i of outward FS Na movement.

The coupling ratio of FS K to Na fluxes was markedly changed when external

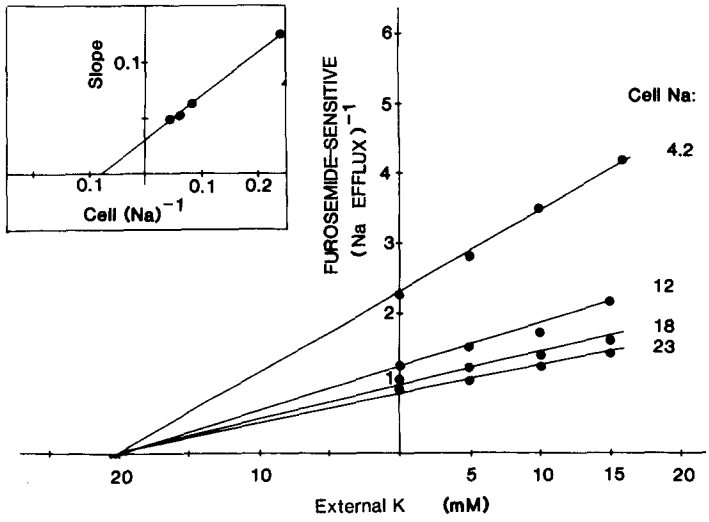

FIGURE 12. Dixon plot for the external K inhibition of FS Na efflux, at four different internal Na concentrations (4, 12, 18, and 23 mmol/liter cell). The inset on the left side plots the slope of each line against the reciprocal of the intracellular Na content. The $K_{0.5}$ for internal Na to stimulate Na efflux in the presence of external K was equal to the one in the absence of external K (13.5 mmol/liter cell). (Subject D.C. Similar results were obtained in the other two subjects.)

K was increased, because the FS Na efflux was inhibited by external K and the FS K efflux was stimulated (Fig. 7). At K_o = 20 mM, the ratio of FS K to Na efflux was 2.3:1. At higher K_o concentrations, the FS Na efflux became almost negligible, whereas there was a significant FS K efflux that was not inhibited by K_o.

The stoichiometric ratio of FS Na and K effluxes is not changed by external Na. Table VI shows the inhibition of FS Na and K effluxes by external Na (130 mM). The effect of external Na was dependent on internal Na, the percentage of inhibition increasing from 20 to 48% when internal Na was increased from 4 to 22 mmol/liter cell.

The statistical analysis (paired *t* test) of the experiments in Table VI showed that there were no significant changes in the stoichiometric ratio of FS Na/K

TABLE V

Kinetic Parameters of the Inhibition by External K of the FS Na Efflux

Subject	$K_{0.5}$ for inhibition by external K	$K_{0.5}$ for activation by internal Na	
		Zero *trans*	K_o *trans*
	mM	*mmol/liter cell*	
D.C.	27.9±0.5	13.4±2.8	13.5±0.3
A.K.	21.5±3.5	13.4±1.2	12.5±0.6
C.B.	20.0±1.6	15.0±1.2	13.5±0.5

The internal Na concentration was varied with the nystatin loading procedure. Under the "zero *trans*" condition, the cells were incubated in an efflux medium containing 150 mM choline Cl. Under the "K_o *trans*" condition, the media contained 150–100 mM choline Cl and 0–50 mM KCl. All media contained (mM): 1 $MgCl_2$, 10 Tris-MOPS, pH 7.4 at 37°C, 10 glucose, and 0.1 ouabain, with and without 1 furosemide. The hematocrit of the efflux medium was 2%. The efflux times were 5 and 65 min. For each subject, the $K_{0.5}$ (± SEM) for inhibition by external K was calculated from the Dixon plot.

effluxes measured in choline as compared with Na media. Therefore, external Na has a different effect than external K on the FS Na and K effluxes. As previously shown by Bize et al. (1982), external Na inhibited FS K efflux when the cells contained both Na and K. In cells containing only K, there was no inhibition of the FS K efflux by external Na.

TABLE VI

Inhibition of the FS Na and K Effluxes from Human Red Cells by External Na

		FS Na efflux		FS K efflux	
	Cell Na	Na medium	Choline medium	Na medium	Choline medium
	mmol/liter cell	*μmol/liter cell·h*		*μmol/liter cell·h*	
A.K.	4	320±15	445±20	405±140	700±65
	12	480±25	770±55	670±150	1,010±40
	23	660±20	1,230±85	830±60	1,350±50
C.B.	4	260±15	320±15	370±100	580±80
	8	410±10	510±25	405±75	800±60
	22	550±25	1,050±70	580±40	1,270±60
D.C.	6	290±10	310±25	—	400±50
	15	425±15	555±30	—	700±65
	17	440±15	730±50	—	800±55

The internal Na and K concentrations were reciprocally varied with the nystatin loading procedure. The cells were incubated in an efflux medium containing (mM): 150 choline Cl (choline medium) or 130 NaCl and 20 choline Cl (Na medium). All media contained (mM): 1 $MgCl_2$, 10 Tris-MOPS, pH 7.4 at 37°C, 10 glucose, and 0.1 ouabain, with and without 1 furosemide. The hematocrit of the efflux medium was 2%. The efflux times were 5 and 65 min. (Fluxes ± SE.)

DISCUSSION

FS K Efflux Can Occur Through Outward Cotransport of Na and K, K_i/K_o Exchange, and Uncoupled K Efflux

We have shown (Fig. 2) that internal Na and K markedly stimulated FS K efflux into choline medium. We interpret this transport route to be an outward cotransport of Na and K. The FS component of K efflux was equal to the Na_i-stimulated K efflux (*cis* stimulation of the OR K efflux). In nitrate medium, there was no *cis* stimulation of OR K efflux by internal Na, but there was an FS component of the K efflux. Thus, Na_i-stimulated and FS K effluxes were greater than the Cl-dependent K efflux.

In cells containing Na and K, incubated in choline media, external K inhibited FS Na efflux, whereas it stimulated FS K efflux and influx. We showed (Fig. 7) that the FS K efflux not mediated by cotransport with Na (extra K efflux) was equal to the FS K influx. Thus, in Na-containing cells, external K may promote a 1:1 K_i/K_o exchange and inhibit the outward cotransport of Na and K.

In the present paper, we also show that in cells containing only K, furosemide does not inhibit K efflux (Fig. 2). However, external K markedly stimulates OR and FS K efflux, without promoting FS K influx (Fig. 5). We called this outward K movement from all K cells the "uncoupled K efflux." In cells containing only K, the uncoupled K efflux is not inhibited by EGTA, but is stimulated by DIDS and Cl removal (nitrate substitution). These results cast doubt upon whether the FS uncoupled K efflux is an operational mode of the Na/K cotransport and require further studies of its coupling to Cl movement.

FS K Influx Occurs Through a K_i/K_o Exchange and Inward Cotransport of Na and K

The present paper provides experimental evidence for the existence of an FS K_i/K_o exchange pathway of the Na/K cotransport, as follows: (*a*) Internal K stimulated the OR and FS K influx (Table III) and there was no FS K influx from choline media into cells containing only Na. (*b*) External K stimulated the OR and FS K efflux (Fig. 7). (*c*) In the absence of external Na, external K stimulated an FS K influx only into Na-containing cells. This influx was a saturable function of external K (Table II and Fig. 6) with a $K_{0.5}$ of 18 mM. (*d*) Internal Na promoted FS K influx from choline media (Fig. 6).

The complete absence of FS K influx into Na-free or K-free cells (Table III) indicates that in order for external K to activate the K influx through the K_i/K_o exchange pathway, both Na and K have to be present on the internal site. The K_i/K_o exchange pathway for the Na/K cotransport can therefore be activated only when there is outward transport of Na and K.

The external site, which promotes activation of the K_i/K_o exchange, also inhibits the FS Na efflux because the stimulation of the K_i/K_o exchange pathway and the inhibition of the FS Na efflux by external K had similar $K_{0.5}$ values (20 mM).

As previously reported by Wiley and Cooper (1974), external Na markedly stimulated OR and FS K influx into fresh cells. This stimulation provides evidence

for the inward cotransport of Na and K. In cells containing only K (Fig. 8*A*), the FS K influx was equal to the Na_o-stimulated component of the OR K influx (inward cotransport of Na and K). When cell Na was increased, the FS fraction of K influx from Na medium increased and was larger than the Na_o-stimulated K influx (Fig. 8*B*). The difference can be accounted for by the stimulatory effect of internal Na on the FS K_i/K_o exchange pathway. Notably, internal Na did not increase the inward cotransport of Na and K (Fig. 9*B*). It seems that the stimulation by internal Na of the FS K influx from Na medium (Fig. 9*A*) is produced through a stimulation of the K_i/K_o exchange pathway rather than of the inward transport of Na/K.

The present experiments therefore show that the FS K influx occurs through two routes: by K_i/K_o exchange and by cotransport of Na and K. Moreover, FS K influx from an isosmotic Na-free medium does not occur through a KCl cotransport but through an Na_i-stimulated K_i/K_o exchange pathway of the Na/K cotransport.

FS Na Efflux Can Occur Through Outward Cotransport of Na and K, Na_i/Na_o Exchange, and Uncoupled Na Efflux

We have shown that internal K stimulated FS Na efflux into choline medium (Fig. 1). However, there was no *cis* stimulation by internal K of the OR Na efflux in the absence of furosemide (Fig. 3). To produce *cis* stimulation, it is of course necessary that OR-FR fluxes remain constant when the *cis* ion substitutions are made. This requirement was not met when the effect of substituting K_i for Na_i on OR Na efflux was measured (Fig. 3). The decrease in the OR and OR-FR Na efflux when internal K was increased masked the *cis* stimulation of the FS Na efflux. The OR-FR Na movement appears, therefore, to be regulated by K and Na through still undefined mechanisms.

Although the OR-FR Na efflux decreased when Na_i was replaced by K_i, the OR-FR K efflux remained constant as Na_i replaced K_i (Fig. 4). The rate constant for OR-FR K efflux into choline medium was 0.013 h^{-1} in all K cells and was reciprocally proportional to K_i as K_i was replaced by Na_i. Garay et al. (1981) showed that at high external Mg, the rate constants for OR-FR Na and K effluxes were similar and independent of the concentration of the other ion. The differences between Garay's observations and the results reported here may be related to the inhibition of Na and K fluxes produced by external Mg, as shown by Ellory et al. (1980, 1983) and Bize et al. (1982). It can be excluded that this behavior is due to the fact that the affinity for furosemide changes with cell Na and K content, because we used maximal inhibitory concentrations (1 mM) of the drug. The reduction of OR-FR Na efflux as Na_i is replaced by K_i is not caused by furosemide, as was observed when only ouabain but not furosemide was present in the medium.

The FS Na influx from K-free medium seems to occur through Na_i/Na_o exchange; therefore, a similar component of the FS Na efflux should also take place through this mechanism.

We have provided evidence for an uncoupled Na efflux in cells containing only Na (Table IV). Na efflux into choline medium was not furosemide sensitive,

but became so in the presence of external Na (130 mM). We proposed to define as "uncoupled Na efflux" the difference between FS Na efflux and FS Na influx in cells containing only Na. The uncoupled Na efflux is responsible for the net FS Na extrusion observed in cells containing only Na incubated in 130 mM NaCl medium (Table IV).

FS Na Influx Can Occur by Inward Cotransport of Na and K and Na_i/Na_o Exchange

The inward transport of Na through Na/K cotransport was estimated from the K_o-stimulated component of the OR Na influx. This component is slightly but significantly lower than the FS component and the difference can be accounted for by the presence of an FS Na influx in the absence of external K, i.e., expression of an FS Na_i/Na_o exchange pathway.

The FS Na_i/Na_o exchange was not inhibited by bumetanide, but it was stimulated by nitrate and this stimulation was inhibited by DIDS. Therefore, we think that this is not an Na_i/Na_o exchange pathway of the Na/K cotransport. Another possibility could be that the FS Na_i/Na_o exchange may represent the effect of furosemide on the phloretin-sensitive Na/Na (Na/Li) exchange system. However, the stimulation by Na_o of the Li efflux is DIDS insensitive, is not stimulated when nitrate is substituted for Cl, and is furosemide insensitive (Canessa et al., 1982).

Variable Stoichiometry of FS Na and K Fluxes

The present study shows that the stoichiometry of the outward FS Na and K fluxes is variable. The stoichiometric ratio between the FS Na and K effluxes varies with the relative ratio of concentrations of Na and K in the *cis* side of the membrane and with the presence of K on the *trans* side.

The stoichiometry of outward FS Na and K movement into choline medium is close to 1:1 when the cells contain equal amounts of Na and K. As shown in Fig. 11, this stoichiometric ratio can vary according to the relative ratio of Na and K content, so that in cells with a low Na/K ratio, the ratio of FS Na/K efflux is <1, whereas in cells with a high Na/K ratio, the FS Na efflux is always larger than K efflux. The stoichiometric ratio of FS Na and K effluxes is therefore determined by the relative ratio of intracellular Na and K concentrations. The variable stoichiometry into choline media can be accounted for by the presence of different binding sites for Na and K ions. In this model, one site has a low $K_{0.5}$ for K, and another site has a low $K_{0.5}$ for Na. A third site has more or less equal $K_{0.5}$ values for Na and K. The relative concentrations of the two solutes will determine the occupancy by Na and K of the different sites for translocation. This model can also account for the asymmetry of the activation curves of Na and K effluxes by internal Na and K (Table I and Figs. 1 and 2). Table VII summarizes the $K_{0.5}$ values for internal and external Na and K to stimulate FS Na and K fluxes. It can be seen that high-affinity and low-affinity binding sites for K and Na are symmetrically distributed on both sides of the membrane.

A variable stoichiometry of FS Na and K effluxes was found not only in choline medium, but also in the presence of external K. The FS Na efflux was inhibited

and the FS K efflux was stimulated by external K (Fig. 7 and Table V). The inhibitory effect of external K on the OR Na efflux is in agreement with previous results described by Sachs (1971), Rettori and Lenoir (1972), Garay et al. (1981), and Haas et al. (1982).

The FS K efflux was stimulated by external K (Fig. 7), as previously observed by Wiley and Cooper (1974). The stoichiometry of FS Na and K effluxes is changed when the external K concentration is increased. This effect is due to the inhibition by external K of the outward cotransport of Na and K and to the stimulation promoted by external K of a K_i/K_o exchange pathway. When the FS Na efflux into choline medium has been almost completely inhibited by external

TABLE VII

Symmetrical Distribution on Both Sides of the Membrane of the Different Affinities for Na and K of the FS Fluxes

Ion	Side	$K_{0.5}$	Reference*
		mM	
K	Outside	4–6	1
	Inside	5	2
K	Outside	20	3
	Inside	20	4
Na	Outside	20–22	5
	Inside	20	6

* (1) From Wiley and Cooper (1974): $K_{0.5}$ for the stimulation by K_o of FS Na influx (Na_o = 150–140 mM) into fresh cells; from Brugnara et al. (1986). (2) Present paper: $K_{0.5}$ for the stimulation of FS Na efflux by K_i in choline medium. (3) Present paper: $K_{0.5}$ for the stimulation by K_o of the FS K influx from choline media. (4) Present paper: $K_{0.5}$ for the stimulation by K_i of the FS K efflux into choline medium. (5) From Wiley and Cooper (1974): $K_{0.5}$ for the stimulation of FS Na influx into fresh cells by Na_o (10–100 mM), with K_o altered reciprocally. (6) Present paper: $K_{0.5}$ for the stimulation of FS Na efflux by Na_i into choline medium; from Garay et al. (1981): $K_{0.5}$ for the stimulation by Na_i of FS Na and K effluxes into 75 mM $MgCl_2$ medium.

K, there is still a sizable FS K efflux, which can be totally accounted for by the K_i/K_o exchange pathway (Fig. 7).

The effect of external K on the FS Na and K effluxes is different from that of external Na. When an inward Na gradient is imposed (130 mM), both Na and K effluxes are inhibited to a similar extent and the stoichiometry is not changed (Table VI).

We showed in the previous paper (Brugnara et al., 1986) that at the equilibrium position of the FS Na and K fluxes in high-K, low-Na cells, the stoichiometry of FS Na and K influxes is 2:3. Under the same conditions, the stoichiometric ratio of the *cis*-stimulated components of the OR Na and K influxes (K_o-stimulated Na influx and Na_o-stimulated K influx, respectively) is close to 1:1. Since the stoichiometric ratio of the *cis*-stimulated influxes is 1:1, the 2 Na:3 K stoichiometry of the FS fluxes is therefore given by the different ratios of K_i/K_o exchange to Na_i/Na_o exchange (Fig. 10). Therefore, when the exchange pathways are not operating, the stoichiometery of Na and K influxes should be close to 1:1. In

cells containing only K (data not shown) at 20 mM external K, the FS and *cis*-stimulated Na and K influxes have similar values.

cis and trans Interactions in FS Na and K Fluxes

We have provided evidence for the presence of several *cis* and *trans* interactions between Na and K. *cis* and *trans* effects are strong indicators of coupling between flows (Heinz, 1978). All these interactions were inhibited by furosemide. Table VIII summarizes our experimental data about *cis* and *trans* interactions between FS Na and K fluxes. The *trans*-inhibitory effect of external Na or Li on the Na and K effluxes was reported by Bize et al. (1982) and Canessa et al. (1982) in

TABLE VIII

cis and trans Interactions Between FS Na and K Fluxes

	$K_{0.5}$ mM
(I) *cis* activating effects	
(1) Na efflux by Na/K cotransport (Stimulation by internal Na)	20
(2) Na influx by Na/K cotransport (Stimulation by external K)	4–5
(3) K efflux by Na/K cotransport (Stimulation by internal Na)	12
(4) K influx by Na/K cotransport (Stimulation by external Na)	—
(II) *trans*-stimulatory effects	
(1) Na efflux by Na/K cotransport (Stimulation by external K in Na medium)	2.5
(2) Na efflux by uncoupled Na efflux (Stimulation by external Na)	—
(3) Na influx by Na/K cotransport and Na_i/Na_o exchange (Stimulation by internal Na)	5
(4) K efflux by uncoupled K efflux (Stimulation by external K)	5
(5) K influx by K_i/K_o exchange	
(Stimulation by internal Na)	8
(Stimulation by internal K)	—
(III) *trans*-inhibitory effects	
(1) Na efflux by Na/K cotransport	
(Inhibition by external K)	20
(Inhibition by external Na)	—
(2) K efflux by Na/K cotransport (Inhibition by external Na)	—

human red cells and by Haas et al. (1982) in duck red cells. The *trans*-stimulatory effects comprise the stimulations by K_o of the Na efflux into Na plus K medium (Brugnara et al., 1986) and of the K efflux (uncoupled K efflux and K_i/K_o exchange), by Na_i and K_i of the K influx (K_i/K_o exchange), and by Na_i of the Na influx (Na_i/Na_o exchange). Even though Haas et al. (1982) initially stated that there were no *trans* effects in the Na/K cotransport, they later observed (Haas et al., 1983) the *trans*-stimulatory effect of internal Na on the K_i/Rb_o exchange pathway.

Reaction Scheme for the FS Na and K Fluxes

We propose to consider the interactions between Na and K in the frame of the reaction scheme shown in Fig. 13. We consider this scheme a minimal model for

the complex coupling phenomena among FS Na and K fluxes. Cl is not considered in this reaction scheme, since we have not yet systematically studied the role of Cl in FS transport of Na and K in human red cells. This remains an important step for the future elaboration of an adequate model. Three forms of the ion complex, with a hypothetical membrane component E, are proposed: a K form, an Na form, and an Na/K form.

This does not indicate that all six modes of operation of the FS Na and K fluxes are mediated by a single membrane component. It is likely that different membrane components are involved in the FS transport, and although the major components (inward and outward cotransport and K_i/K_o exchange) take place

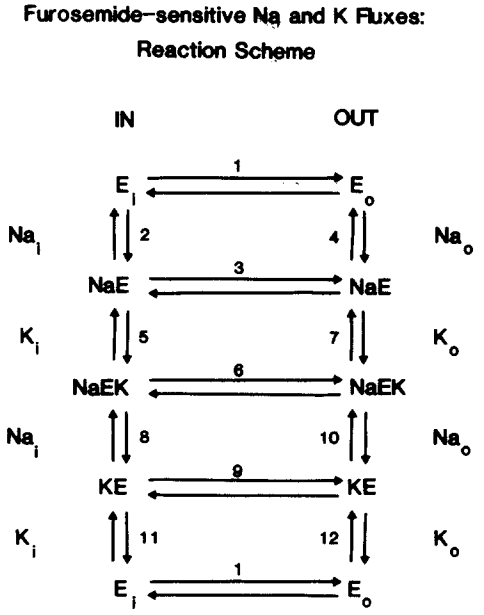

FIGURE 13. Reaction scheme for the FS Na/K cotransport.

through an Na/K/Cl cotransport system, other components (Na_i/Na_o exchange and uncoupled K efflux) probably take place through different pathways.

Inward and outward cotransport can be accounted for by reaction 6. The inhibition by external K of FS Na efflux, but not of FS K efflux into choline media, can be explained by considering that K comes off first and then Na at the external side. External K allows K but not Na to be released from the ENaK form; therefore, Na is trapped in an Na-loaded form, which does not return in the presence of external K, whereas the EK form returns loaded with K (K_i/K_o exchange).

Reaction 9 may account for the K_i/K_o exchange mode. The dependence on internal Na can be explained by the coupling of reaction 9 with reactions 6–8. Alternatively, it can be accounted for by a combination of reactions 5, 8, and 9. Reaction 3 may account for the Na_i/Na_o exchange mode. External Na can inhibit

both Na and K effluxes, if, in the absence of external K, Na_o shifts reaction 7 or reaction 10 to NaEK.

An additional loop should be considered to explain the uncoupled K efflux: KE should be able to translocate (reaction 9) and return as the unloaded form E (reaction 12) in the presence of external K. A similar loop could also explain the uncoupled Na efflux.

Modes of Operation of the FS and OS Na and K Fluxes

We would like to compare briefly the *cis* and *trans* interactions between Na and K ions transported by the FS Na/K fluxes and the OS Na/K pump in human red cells. Both transport systems have several modes of operation: outward and inward, or forward and backward, uncoupled efflux, and Na_i/Na_o and K_i/K_o exchange. In Table IX, several modes of operation of the Na/K pump (Glynn

TABLE IX

Comparison of the Different Modes of Transport in the FS and OS Pathways

	FS transport	OS Na/K pump
(I) Net Na extrusion	K_o inhibition	K_o stimulation
	Na_i stimulation	Na_i stimulation
	Na_o inhibition	Na_o independent
(II) Na_i/Na_o exchange	—	K_o inhibition
	Na_i stimulation	Na_i stimulation
	Na_o stimulation	Na_o stimulation
(III) K_i/K_o exchange	Na_i stimulation	Na_i inhibition
	Na_o independent	—
	K_i, K_o stimulation	K_i, K_o stimulation
(IV) Uncoupled K efflux	K_o stimulation	—
(V) Uncoupled Na efflux	Na_o stimulation	K_o inhibition
	—	Na_o inhibition
	—	Na_i stimulation
(VI) Reversal	—	Na_i inhibition
	—	K_o inhibition
Stoichiometry under physiological conditions	3 K:2 Na	3 Na:2 K
Free energy available	Na, K, and Cl gradients	ATP hydrolysis

and Karlish, 1975) and the FS Na and K transport are compared. The *cis* and *trans* interactions between Na and K are also very different. The K_i/K_o and Na_i/Na_o exchange pathways of the Na pump are inhibited by internal Na and external K, respectively, and the K_i/K_o exchange of the Na/K cotransport is stimulated by internal Na. The net Na extrusion through the Na pump is *trans*-stimulated by external K, whereas in the Na-K cotransport, it is *trans*-inhibited. In the absence of external K, two-thirds of the pump can operate in the Na_i/Na_o exchange mode. In the absence of external Na, external K can bring Na/K cotransport to operate 100% in the K_i/K_o exchange mode.

Although in the Na pump the net flux of Na and K is coupled to the ATP hydrolysis, with a large change in free energy ($\Delta G^{\circ\prime} = 14$ kcal), the FS Na and K fluxes can be driven by the sum of the free energy available from the chemical

gradient of Na, K, and Cl ($\Delta G^{\circ\prime} = 1.4$ kcal). Although the Na/K pump has a high K_m for ATP, only when ATP levels are lowered below 100 μmol/liter cell by metabolic depletion is there a significant inhibition of the FS fluxes (Dagher et al., 1985); this seems to indicate that the systems are coupled to metabolism by different mechanisms.

In the present paper, we have provided experimental evidence for several FS modes of transport of Na and K. The coupling of Na and K movements occurs through *cis*-stimulatory, *trans*-stimulatory, and *trans*-inhibitory interactions. Most of these interactions take place through an Na/K cotransport system, which can operate with a variable stoichiometry according to the relative ratio of Na and K concentrations on both sides of the red cell membrane.

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